Novel Regulation of Cortical Acetylcholine Release and Cognitive Behavior

A Senior Honors Thesis

Presented in Partial Fulfillment of the Requirements for graduation *with research distinction* in the undergraduate colleges of The Ohio State University

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DEDICATION

I wholeheartedly dedicate this work to my husband, David, my parents, and the rest of my family for their continued care, love, and encouragement throughout my life.

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ABSTRACT

There is an evolving literature demonstrating the ability of non-neuronal cells to regulate the release of traditional neurotransmitters within various brain regions. Specifically, astrocytes may play a more substantial role in regulating chemotransmission than previously thought. Anatomically, astrocytes lie in close apposition to neurons and can functionally impact neuronal signaling through generation of neuroactive compounds like kynurenic acid (KYNA), a byproduct of the kynurenine pathway (KP) of the metabolism of tryptophan. Endogenous levels of KYNA antagonize _7 nicotinic ACh receptors (nAChR). As such, KYNA can regulate the release of traditional neurotransmitters like glutamate and dopamine which have the ability to positively modulate acetylcholine (ACh) levels in the medial prefrontal cortex (mPFC). Given the essential activity of nAChR in cognitive behaviors such as attentional processing, dysregulations in the KP may be functionally significant as elevated levels of cortical KYNA have been associated with schizophrenia. Furthermore, schizophrenic patients also display severe cognitive deficits in executive functioning characterized by impaired cognitive flexibility. Performance in tasks that measure cognitive flexibility, such as perceptual set shifting, is dependent upon the integrity of prefrontal activity. As such, this project has two specific aims. The first determines the ability of local KYNA to modulate basal cortical ACh release in the mPFC. Male Wistar rats were implanted with microdialysis guide cannulae into the mPFC, and the rats were perfused, in randomized order, with the following drug treatments; artificial cerebral spinal fluid (aCSF) as a vehicle control, KYNA (100 nM), a kynurenine aminotransferase II (KAT II) inhibitor, UPF-874 (5 mM), and KYNA (100 nM) + UPF-874 (5 mM). We discovered that KYNA bi-directionally modulates basal ACh release in the mPFC as KYNA attenuated ACh release and inhibition of its synthesis by UPF-874 potentiated ACh release locally within the mPFC. The second aim determined whether varying KYNA levels was associated with a type of cognitive deficit observed in schizophrenia. Male Wistar rats were tested in a

prefrontally-mediated set-shifting task which required two session of testing on consecutive days. On day 1 (Set 1), animals are trained to discriminate between brightness (black versus white) or texture (smooth versus rough) dimensions, until reaching a criterion performance level (8 consecutive correct trials). On day 2 (Set 2), animals are trained on the alternative (extra-dimensional) discrimination strategy for 80 trials, regardless of performance. Animals received either a systemic injection of saline or kynurenine (50 mg/kg) prior to testing on Set 1 or Set 2. We discovered that elevated KYNA levels specifically impaired the ability of animals to perform an EDS that was not due to a general cognitive impairment as increasing levels of this metabolite did not impair rule acquisition. Collectively, these studies indicate a role for KYNA dysfunction in schizophrenia. Therefore, drugs which inhibit or decrease the production of KYNA (ie. KAT II inhibitors) may be effective pharmacotherapies for the treatment of cognitive deficits observed in schizophrenia.

INTRODUCTION

1.1 Schizophrenia

Schizophrenia is one of the most severe and debilitating neuropsychiatric disorders and may affect up to 1% of the general population (Carpenter and Buchanan, 1994). Although significant advances have been made both in terms of understanding its pathophysiology and treatment strategies, the prognosis for schizophrenia is poor and treatment remains a major challenge in mental health care. This may be partially explained by the fact that schizophrenia is associated with a multiplicity of symptoms including positive symptoms, negative symptoms, and cognitive dysfunction (Andreasen, 1995). Positive, or psychotic, symptoms include hallucinations, delusions, and thought disorders whereas negative symptoms include a flattened affect or social withdrawal. In addition, patients present with severe cognitive impairments as many domains of cognition are differentially disrupted, one of which includes executive functions like attention and cognitive flexibility (Andreasen, 1995). This aspect of the schizophrenia is critical because these impairments often accompany (Saykin et al., 1994) or precede (Fuller et al., 2002) the onset of psychosis, and remain throughout the course of the illness (Green & Braff, 2001). More importantly, the severity of cognitive dysfunction is positively correlated with the decline in quality of life of patients with schizophrenia (Green et al., 1996). Antipsychotic medication is a standard treatment strategy for individuals diagnosed with the disease. The use of newer atypical antipsychotics is commonplace, and while they demonstrate increased efficacy treating positive and negative symptoms with fewer extrapyramidal side effects, they do not completely alleviate the neurocognitive deficits (Peuskens et al., 2005). Therefore, understanding the regulation of neural systems that are involved in cognitive functioning is critical for the development of more efficacious pharmaceutics to normalize the cognitive impairments observed in schizophrenia.

1.2 Basal Forebrain Cholinergic System and Regulation of Acetylcholine

One neural system whose activity has been demonstrated to be essential in normal attentional processing, which is a necessary early mediator of cognitive function (Sarter & Bruno, 1997), is the basal forebrain cholinergic system (BFCS). The integrity of attentional processing is mediated by acetylcholine (ACh) innervation of the prefrontal cortex (PFC) that is supplied from the cholinergic neurons originating from the nucleus basalis of Meynert / substantia innominata (SIO) region of the BF (Figure 1; Sarter et al., 2005). In fact, impairments in task performance are evident following selective lesions of basal forebrain cholinergic neurons projecting to the cortex (Dalley et al., 2004). Furthermore, these projections terminating in the PFC are necessary for the expression of executive functions like attention and cognitive flexibility (Birrell & Brown, 2000). It has been proposed that aberrations within this system may contribute to the cognitive deficits observed in various neuropsychiatric disorders (Sarter & Bruno, 1999). As such, the identification of regulatory mechanisms mediating ACh transmission is increasingly relevant to understand and effectively treat the cognitive deficits associated with schizophrenia.

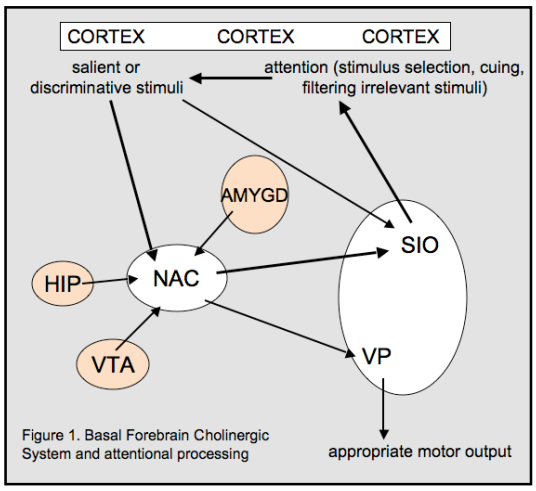

Figure 1. Basal Forebrain Cholinergic System and attentional processing

1.3 Non-Neuronal Modulation of Chemotransmission

Previously, the regulation of chemotransmission exclusively focused on the modulation by other traditional neurotransmitter systems. For instance, it has been demonstrated that two neurotransmitters, dopamine (DA) and glutamate (Glu) are capable of positively modulating ACh release in the PFC (LaPlante et al., 2004; Del Arco et al., 2007). Additionally, this regulatory capacity also extends to the distal, yet functionally

related nucleus accumbens (NAC) (Figure 1). This brain region is relevant because afferents that synapse in the BF release the inhibitory neurotransmitter γ-aminobutyric acid (GABA). Therefore, these projections from the shell region of the NAC act as a critical modulator of BFCS excitability. Dopamine (DA) and glutamate (Glu) interactions regulate the excitability of these NAC projections (Ariano et al., 1997) and, as a result, the activity of the BFCS and ACh levels in the PFC. Therefore, it has been shown that local pharmacological manipulations in the NAC are capable of trans-synaptically regulating cholinergic transmission in the distal, yet functionally related, PFC as well as locally regulating cortical ACh transmission.

1.4 Kynurenine Pathway

In contrast to the regulation of chemotransmission by other neurotransmitter systems and other brain regions, it is becoming increasingly evident that astrocytes and other non-neuronal support cells may play a more substantial role in neuronal communication than indicated by their typical biochemical supportive functions. Anatomically, astrocytes lie in close apposition to neurons (Haydon, 2001) and can functionally impact neuronal signaling through the generation of neuroactive compounds like kynurenic acid (KYNA), a metabolite of the kynurenine pathway (KP) of tryptophan degradation (Figure 2; Schwarcz et al., 2001). Initially, KYNA was reported to be a broad-spectrum

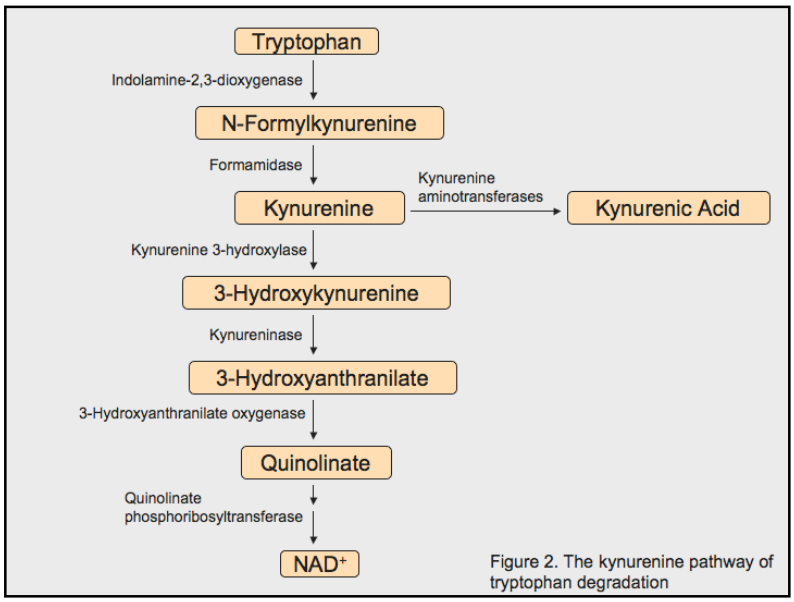

Figure 2. The kynurenine pathway of tryptophan degradation

antagonist of excitatory amino acid (EAA) receptors, it was later discovered that the metabolite possessed neuroprotective properties mediated through inhibition of the glycine co-agonist (glycine$_B$-receptor) of the NMDA receptor (Foster et al., 1984). However, KYNA must compete with glycine, which has an approximately 20x higher affinity for the receptor than KYNA (Parsons e al., 1997), and D-serine, a potent agonist of the site (Schell, 2004), for access to the glycine$_B$-receptor. At nanomolar concentrations, this metabolite is also a potent, non-competitive antagonist of _7 nicotinic acetylcholine receptor (_7nAChR; Hilmas et al., 2001). The previous data, in conjunction with other studies, provides much evidence for the proposition that _7nAChR rather than the glycine$_B$-receptors are the primary *in vivo* target of nanomolar concentrations of KYNA in the striatum (Alkondon et al., 2004; Hilmas et al., 2001; Wu et al., 2007).

1.5 Kynurenic Acid and Regulation of Neurotransmitter Release

As KYNA is a natural constituent in the mammalian brain it may play a role in brain physiology, and especially pathology, as KYNA is an antagonist of both the NMDA receptor (Schwarz & Pellicciari, 2002) and the _7nAChR (Hilmas et al., 2001). The fact that KYNA is capable of interacting with both of these Ca^{2+} permeable receptors suggests a physiological function in glutamatergic and cholinergic neurotransmission. Interestingly, dysregulations in the KP have been implicated in various disease states that are also characterized by glutamatergic and cholinergic abnormalities (Nemeth et al., 2005). In fact, direct support for such a regulatory role has been provided by *in vivo* studies in the rat striatum where a reduction in KYNA levels enhances vulnerability to an excitotoxic insult (Poeggeler et al., 1998), and conversely, modest elevations of KYNA inhibit glutamate release (Carpendo et al., 2001). Furthermore, changes in KYNA levels also influences another neurotransmitter, DA, as comparatively modest increases in KYNA reduces extracellular DA levels (Rassoulpour et al., 2005) while reductions elevate extracellular levels (Wu et al., 2007).

Since both DA and Glu positively modulate ACh levels, KYNA may influence BFCS activity by negatively modulating ACh release (Del Arco et al., 2007; LaPlante et al., 2004).

1.6 Biochemistry of Kynurenine Pathway

Therefore, given the ability of KYNA to regulate both DA and Glu release, and its potential to influence ACh transmission, understanding the fundamental principles underlying its synthesis is essential. While endogenous levels of KYNA are influenced by metabolic processes (Hogkins et al., 1998), they are primarily controlled by the bioavailability of its precursor L-kynurenine (L-KYN), which readily crosses the blood brain barrier from the peripheral circulation. Although the majority of L-KYN is peripherally derived, enzymes responsible for its formation are also located in the brain (Guidetti et al., 1995). The precursor is then actively transported into astrocytes by a sodium-independent transporter for large neutral amino acids (Speciale, 1989); however, a less efficient, sodium-dependent mechanism also transports KYN into neurons (Speciale & Schwarcz, 1990). Subsequently, KYNA is formed through the irreversible transamination of KYN by kynurenine aminotransferases (KATs) I and II (Okuno et al., 1991). KAT II, which is preferentially responsible for the *de novo* synthesis of KYNA as this enzyme operates best at physiological pH and preferentially recognizes L-KYN as a substrate (Guidetti et al., 1997, 2007a), is primarily localized in astrocytes as demonstrated by immunocytochemical studies (Guidetti et al, 2007b). After synthesis, KYNA is rapidly liberated into the extracellular space through a calcium-independent process (Turski et al., 1989), but the precise mechanism has not been elucidated. Interestingly, KYNA cannot be removed from the extracellular space by re-uptake or enzymatic degradation (Turski & Schwarcz, 1988) so transport by a non-specific probenecid-sensitive process appears to be the only mode by which KYNA is eliminated from the brain (Moroni et al., 1988). In accordance, probenecid

adminisration to rats is associated with elevated concentrations of endogenous kynurenic acid in the brain (Erhardt et al., 2002).

Obviously, this pathway can be manipulated to vary endogenous levels of KYNA in order to test hypotheses. For instance, direct perfusion of KYNA or increasing precursor levels of L-KYN would elevate KYNA levels. In contrast, interfering with the biosynthetic machinery of its synthesis through inhibiting KAT II would decrease levels of KYNA.

1.7 Kynurenic Acid and Schizophrenia

Considering the previous evidence that demonstrates a physiological role for KYNA, such as the modulation of other neurotransmitter systems and its preferential inhibition of _7nAChR, manipulations of the KP would be a useful method to elucidate the mechanisms mediating its biological function(s). Given the essential activity of _7nAChR in cognitive behaviors such as attentional processing (Dani & Bertrand, 2007), dysregulations in the KP may be functionally significant. Interestingly, elevated levels of cortical KYNA have been associated with schizophrenia and the increases were seen only in the prefrontal cortex, a cortical area traditionally thought to be involved in the pathophysiology of the disorder (Schwarcz, 2001). Furthermore, schizophrenic patients also display severe cognitive deficits in executive functioning characterized by impaired cognitive flexibility (Ragozzino, 1999). This refers to a humans' or animals' ability to shift behavioral response strategies when there is a change in environmental contingencies. Performance in tasks that measure cognitive flexibility, such as perceptual set-shifting, therefore requires the organism to shift attention to different dimensions of the stimulus array based on changes in rules or rewards encountered during the course of a problem-solving task (Owen et al., 1991). Perceptual set-shifting in humans is typically assessed using variants of the Wisconsin Card Sorting Task (WCST; Robbins, 2007). Extensive evidence indicates that individuals with schizophrenia and other patients with PFC damage perform poorly on the WCST (Pantelis

et al., 1999). The maze-based set-shifting task was developed to reveal many of the same cognitive operations highlighted by the WCST (Birrell & Brown, 2000). There is considerable evidence in both primates (Dalley et al., 2004) and rats (Birrell & Brown, 2000) indicating that the integrity of prefrontal function strongly influences the quality of performance in set-shifting tasks. Additionally, discrimination amongst novel, complex stimuli is more rapid when the discrimination rule is based on the same perceptual dimension (i.e. intradimensional shift, ID) rather than to a different perceptual dimension that was previously irrelevant to solving the task (i.e. extradimensional shift, EDS; Birrell & Brown, 2000). As such, performance deficits in the WCST by schizophrenic patients typically reflect reduced performance on the EDS component of the task (Jazbec et al., 2007).

1.8 Hypotheses

Overall, given the ability of KYNA to negatively modulate neurotransmitter systems that positively modulate ACh transmission, and based on data implicating elevations in KYNA in the pathophysiology of schizophrenia, we propose KYNA may have a regulatory capacity over the BFCS as well. We therefore, conducted two experiments to test the role of KYNA in the regulation of cortical ACh release and cognitive behavior. The first experiment tested the capacity of KYNA to modulate basal ACh transmission in the medial prefrontal cortex (mPFC) and then extended this modulation to the role of endogenous tonic concentrations of KYNA. The second experiment used a set-shifting task as a measure of cognitive flexibility to reveal a role for KYNA in the regulation of prefrontally-mediated cognitive behavior. We propose that if the astrocyte-derived metabolite KYNA has a critical role in the regulation of basal ACh transmission, then changes in extracellular levels of KYNA will modulate ACh release locally in the PFC. Furthermore, as schizophrenics present with impairments in cognitive flexibility, an executive function mediated by

prefrontal activity, and elevated KYNA levels, increases in this metabolite may result in impaired flexible responding.

MATERIALS AND METHODS

1. SUBJECTS

Male Wistar rats (Charles River Labs, Wilmington, MA, USA) weighing between 300-400 grams were utilized for all studies. Animals were maintained on a 12:12 hour light:dark cycle (lights on: 0600) in a temperature- and humidity-controlled room. Animals were individually housed in plastic cages lined with corn cob bedding (Harlan Teklad, Madison, WI, USA) and had access to food and water *ad libitum*. All procedures involving animals were approved by The Ohio State University Institutional Animal Care and Use Committee in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Three days prior to surgery, animals were acclimated to the microdialysis testing environment in clear plastic bowls (35 cm height x 38 cm diameter; CMA, Stockholm, Sweden) lined with corn cob bedding. Animals remained in the bowls for a minimum of four hours/day, and returned to their home cages at the end of each acclimation period.

2. SURGERY

Following their three days of acclimation, animals were anesthetized using isoflurane gas (2%, 0.6 L/min, O_2 delivery) and unilaterally implanted with microdialysis guide cannula (0.38 mm o.d.; Sci Pro Inc., Sanborn, NY, USA) into the mPFC (A = 4.2, L = ±0.6, V = 0.6 mm, 20º rostral angle relative to Bregma and dura). All guide cannula are fixed to the skull using stainless steel screws and dental acrylic. Stylets, ending flush with the guide cannula, were inserted to prevent cannula occlusion. The surgical site was swabbed with a topical antibiotic ointment (lidocane, 5%), and animals also received a

prophylactic dose of the antibiotic chloroamphenicol (100 mg/ml; 0.125 ml) administered subcutaneously. Following surgery, animals were returned to their home cages and allowed to recover for 4 days prior to microdialysis testing or resuming behavioral training.

3. GENERAL MICRODIALYSIS PROCEDURES

Microdialysis sessions were conducted using repeated perfusions, with each animal receiving four different pharmacological manipulations, one every other day. This repeated testing paradigm has the advantage of decreasing variability among treatment conditions because each subject serves as its own control. Furthermore, it allows for paradigms such as dose-response analyses, as well as agonist-antagonist interactions to be studied in the same animal. The procedure has been repeatedly validated by demonstrating that basal cortical ACh efflux does not significantly change over repeated dialysis sessions, and that the effects of behavioral, pharmacological, or sensory manipulations on ACh levels do not interact with dialysis sessions (Bruno et al., 1999; Nelson et al., 2002).

On the fourth day following surgery, animals were brought to the testing environment and allowed to acclimate for 30 minutes prior to the insertion of microdialysis probes. Following the 30-minute acclimation period, stylets were removed and probes (Sci Pro, Inc., 0.2 mm o.d., 3 mm membrane tip for PFC) were inserted into each guide. Inlet and outlet lines for the dialysis probes were attached to a two-channel liquid swivel (Instech, Plymouth Metting, PA).

Probes were continuously perfused with artificial cerebral spinal fluid (aCSF) (containing in mM: NaCl 166.5, $NaHCO_3$ 27.5, KCL 2.4 $CaCl_2$ 1.2, Na_2SO_4 0.5, KH_2PO_4 0.5, glucose 1.0, pH 7.1) at a flow rate of 1.25 μl/min. No acetylcholinesterase inhibitor was utilized in any experiment. A three-hour wash out period was observed after probe insertion to allow ACh efflux to reach a stable baseline that was maximally sensitive to TTX

before beginning collections (Moore et al., 1992). All microdialysis experiments conducted followed this general paradigm.

Microdialysis for animals participating the set shifting tests will be conducted in a similar manner except all post-surgical acclimation will be done in the maze itself and on each day of testing, animals will be placed into the start arm and barricaded there for a 2-hour wash-out period prior to task onset. Artificial CSF will be perfused throughout the sessions and ACh will be collected every 8 minutes (flow rate: 2.0 µl/min) once the task begins.

4. HPLC Analysis

Acetylcholine

Dialysis samples were stored at -80º C until analyzed using high performance liquid chromatography (HPLC) with electrochemical techniques. A volume of 15 µl of each sample was injected by an autosampler (ESA Inc., Chelmsford, MA). ACh and choline were separated by a UniJet microbore analytical column (BAS Inc.; 1 x 50 mm) using a sodium phosphate mobile phase (35 mM Na_2HPO_4, 0.36g EDTA, 1.0% of the microbicide Proclin, pH = 8.5), flowing at 0.15 ml/min. A post-column IMER containing acetylcholinesterase and choline oxidase was used to break down ACh into H_2O_2 (Potter et al., 1983), and quantified using a peroxidase-wired glassy carbon electrode.

Kynurenic acid

KYNA levels were analyzed using HPLC with florescence detection (excitation wavelength: 344nm; emission wavelength: 398nm) by our collaborators at the University of Maryland (Wu et al., 2007).

5. HISTOLOGY

Following the final microdialysis session, animals were given an overdose of sodium pentobarbital and trans-cardially perfused with 0.9% heparinized saline followed by 10% formalin. Brains were removed and stored in 10% formalin for at least 24 hours, and were then transferred to a 30% sucrose solution for three days. Brains were sectioned using a cryostat and sections (50 μm) were mounted on gelatin-coated slides, stained using Cresyl Violet, and examined under a light microscope. Subjects whose probe and injection cannula placements were located outside any of the targeted regions were excluded from further analysis.

6. DATA ANALYSIS

Changes in basal ACh efflux (fmol/15 μl) across sessions and treatment groups were analyzed using one-way repeated measures analysis of variance (ANOVAs). Basal efflux was then defined as the mean of the four baseline collections, and subsequent data were expressed as percent change from that mean baseline. Statistical analysis of drug effects was conducted using a two-way, within-subjects ANOVA with drug GROUP and TIME as within-subjects measures. Significance was defined as $P < 0.05$, and the Huynh-Feldt correction was utilized to reduce Type I errors associated with repeated measures ANOVAs (Vasey and Thayer, 1987). All statistical tests were preformed using SPSS for windows (version 14.0).

7. ATTENTIONAL SET-SHIFTING

APPARATUS

The set-shifting maze (Figure 3) is constructed out of pressed wood (0.7 cm thick) and is sealed with a polyurethane primer. The maze is composed of a central platform (14 cm each side) and four arms (40 cm each) radiating from the central platform. The arms

are 14 cm wide with walls 20 cm in height. A hexagonal plastic food dish was placed at the end of each arm and was sufficiently deep where a food reward could not be seen when entering the arm. Maze arms varied along two stimulus dimensions: brightness and texture. Two arms were covered with a faux leather material in black and the other two were covered in the same material in white. The smooth texture (on the floor) consisted of the faux leather material covering the walls, and the rough texture consisted of rough grade sandpaper spray painted either black or white to correspond with the appropriate colored arms. Arm combinations were: white/smooth, white/rough, black/smooth and black/rough. The maze was attached to a rotary platform. The holding cage used for inter-trial intervals was a standard rat housing cage lined with corn-cob bedding.

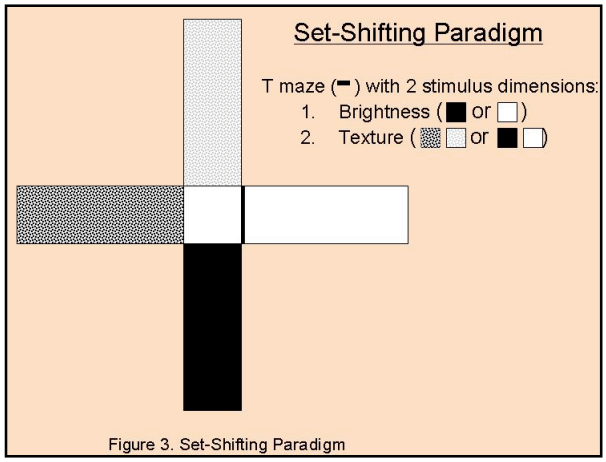

Figure 3. Set-Shifting Paradigm

SET-SHIFTING TRAINING

All training and testing paradigms were modeled after procedures used by Stefani and Moghaddam (2005a). Animals were first food restricted (given 15-20 g/day) and maintained at a minimum of 85% of their free-fed weight. During the pre-acclimation phase, animals were given a food reward consisting of a sugary cereal (Froot Loops) in their home cages to familiarize them with the taste and odor of the reward, and to reduce possible neophobic effects of reward introduction in the maze. Following a week of handling and food restriction, animals began one of two phases of maze acclimation. During the first day of acclimation, multiple animals were placed in the maze and were allowed 10 minutes to explore and consume any food rewards there. Following this first day, animals were individually placed in the maze in which all four food wells were baited with pieces (1/3 of

an individual cereal piece) of Froot Loops. Rats were given 10 minutes to roam freely and explore the apparatus, to condition the rats to receiving food rewards while in the maze. On subsequent days of acclimation, each food well was baited with one piece of Froot Loop per well, and rats were allowed to explore until all food had been consumed, up to 10 minutes. Upon removal from the maze, rats were briefly placed in a holding cage before returning to their home cages. Surgical implantation (see above) of injection and microdialysis guide cannulae were done following this first phase of acclimation.

During the second phase of acclimation, the maze was set up in a T configuration (with a divider placed to block one arm). Animals received 8 trials per day, consisting of two starts from each arm. Animals were placed in the central arm, allowed to turn left or right (choosing one arm) and consume any food reward there. Arms were reinforced randomly. Between trials, rats were removed from the maze and placed in a holding cage (inter-trial interval approx. 15 s).

SET-SHIFTING TESTING

The attentional set-shifting task consists of two sessions on consecutive days, with the maze in a T configuration as it was during the second phase of acclimation. On day 1 (Set 1) animals were trained to discriminate between brightness (black verses white arms) or texture (smooth verses rough arms) dimensions, and were trained to reach a criterion performance level consisting of 8 consecutive correct trials. Animals were allowed 2 minutes to make a 'choice' by which an animal had to leave the start arm and enter one choice arm far enough so all four feet passed into the choice arm. Animals were allowed up to 120 trials to reach criterion, and animals who failed to learn the initial discrimination following 120 trials were removed from study. On day 2 (Set 2), animals were trained on the alternative (extra-dimensional) discrimination strategy for 80 trials, regardless of performance level (Stefani and Moghaddam, 2005a).

SET-SHIFTING DATA ANALYSIS

All statistical analyses were conducted according to tests described in Stefani and Moghaddam (2005a). Dependent measures including trials and time to criterion (Set 1) and time to criterion (Set 2) were analyzed using one-way analysis of variance (ANOVA). Because the trials to criterion for Set 2 were restricted under a cap of 80 trials (Stefani and Moghaddam, 2005a), these data were analyzed using the non-parametric Kruskal-Wallis test with Mann-Whitney post hoc testing. The percentage of correct scores was analyzed for each 8-trial block (over 10 consecutive blocks) to examine performance across blocks of trials for Set 2. Performance across trial blocks was analyzed using mixed-design 2-way, repeated measures ANOVAs, with drug treatment as the between subjects variable and trial block as the within subjects measure (Stefani and Moghaddam, 2005a). Analysis of perseverative responding during Set 2 was conducted by comparing the percent correct scores from each of two start-arm designations (perseveration and reinforcement arms) within each block of trials. Perseveration arms consisted of the start arms that yielded an incorrect response during Set 2, but were previously reinforced during Set 1. Reinforcement arms were designated as those yielding a correct response during Set 2 when responding according to the strategy that was successful during Set 1. Perseverative arm and reinforcement arm performance was analyzed by comparing percent correct scores using mixed design 2-way, repeated measures ANOVAs with treatment group as the between-subjects and trial block as the within-subjects variables. Using this paradigm, animals can make three kinds of errors in learning the new strategy for Set 2. These include perseverative errors where animals choose the previously reinforced dimension. For example, animals that learned to choose the *black* arm during Set 1 but need to learn the *smooth* arm for Set 2 would be making perseverative errors if they repeatedly chose the black/rough (incorrect) arm over the white/smooth (correct) arm. Animals may also make errors in which they fail to learn the new strategy; these errors include entry into never-

reinforced arms which includes the white/rough arm in this example. Animals may also make regressive errors in which animals begin to adopt the new (smooth) strategy, but then revert back to the original rule from Set 1 (Floresco et al., 2006).

RESULTS

Guide cannulae placements

All placements were located in the mPFC. Figure 1 shows a representative placement of this area. Any animals whose probe placements fell outside of the mPFC were excluded from further analysis.

Manipulation of the KYN pathway to decrease endogenous levels of KYNA

This manipulation validated the ability of a KAT II inhibitor, UPF-874, to subsequently decrease KYNA in the mPFC. Figure 2 illustrates the effects of 5 mM UPF-874 on KYNA efflux (n = 5). Administration of UPF-874 significantly decreased KYNA levels across collections (TIME, $F_{11,44} = 8.643$, $P < 0.001$). Data from this experiment was gathered from our collaborators at the University of Maryland (unpublished observations, Wu, H.Q. and Schwarcz, R.).

Ability of KYNA to regulate basal cortical ACh transmission

This experiment examined the contribution of physiologically-relevant concentrations of KYNA to the basal regulation of cortical ACh release. Figure 3 illustrates the effects of aCSF-vehicle, 100 nM KYNA, 5 mM UPF-874, and 100 nM KYNA + 5 mM UPF-874 on cortical ACh efflux (n = 7). Basal levels of ACh efflux remained stable over the four dialysis sessions (SESSION, $F_{3,18} = 0.808$, $P = 0.474$), and across all drug treatments (DRUG, $F_{3,18} = 3.181$, $P = 0.077$), as revealed by one-way ANOVAs. Basal levels of ACh

(mean ± S.E.M., fmol/15 µl) were 3.1 ± 0.8, 5.2 ± 0.7, 2.7 ± 0.3, and 5.4 ± 1.1 for aCSF, 100 nM KYNA, 5 mM UPF-874, and 100 nM KYNA + 5 mM UPF-874 sessions, respectively. Given that basal levels of ACh efflux did not differ over session or group, all subsequent values were expressed and analyzed as a percent change from session baseline. Intra-cortical perfusion of drugs led to differential effects on cortical ACh efflux (DRUG, $F_{3,18}$ = 29.080, P < 0.001). These effects were found to vary across collection interval (TIME $F_{10.60}$ = 3.638, P = 0.001; DRUG x TIME, $F_{30,180}$ = 2.787, P = 0.002). Considering the overall main effects comparing all four treatment groups, a series of smaller 2-way ANOVAs were conducted to look for differences between treatment groups.

Administration of 5 mM UPF-874 significantly increased ACh levels above the vehicle aCSF session (DRUG, $F_{1,6}$ = 44.826, P < 0.001; TIME, $F_{10,60}$ = 4.899, P < 0.001; DRUG X TIME, $F_{10,60}$ = 2.930, P = 0.032). UPF-874 was significantly higher than aCSF within the first 15 minutes of perfusion (collection 5, t_5 = -3.773, P =0.009). In addition, administration of UPF-874 significantly increased ACh levels above KYNA (DRUG, $F_{1,6}$ = 50.520, P < 0.001; TIME, $F_{10,60}$ = 3.186, P = 0.004; DRUG X TIME, $F_{10,60}$ = 5.358, P < 0.001). UPF-874 was significantly higher than KYNA within the first 15 minutes of perfusion (collection 5, t_5 = -3.887, P =0.008). Furthermore, co-perfusion of UPF-874 and exogenous KYNA was able to attenuate the UPF-874–mediated increases in ACh as levels between UPF-874 and the combination treatment were significantly different (DRUG, $F_{1,6}$ = 67.171, P < 0.001; TIME, $F_{10,60}$ = 4.595, P < 0.001; DRUG X TIME, $F_{10,60}$ = 1.020, P = 0.003). UPF-874 was significantly higher than co-perfusion of both drugs within the first 15 minutes of perfusion (collection 5, t_5 = 5.093, P =0.002). Interestingly, this highlights the role of astrocytic activity in the modulation of ACh transmission.

There was a significant effect of DRUG between the administration of 100 nM KYNA and the combination treatment of KYNA and UPF-874 ($F_{1,6}$ = 6.369, P = 0.045), but there

was no effect for TIME ($F_{10,60}$ = 0.925, P = 0.517) or DRUG X TIME ($F_{10,60}$ = 1.020, P = 0.436).

Overall, these results suggests that KYNA, and hence astrocytic activity, negatively modulates PFC ACh release. KYNA also *tonically* inhibits *basal* ACh release as the KAT II mediated reduction in KYNA synthesis markedly increases cortical ACh.

Manipulation of the KYN pathway to increase endogenous levels of KYNA

This manipulation validated the ability of increased precursor levels, L-KYN, to subsequently increase KYNA levels in the mPFC. Figure 4 illustrates the effects of a systemic injection of L-KYN (50mg/kg) on KYNA efflux (n = 5). Administration of L-KYN significantly increased KYNA levels across collections (TIME, $F_{15,45}$ = 124.825, P < 0.001). Data from this experiment was gathered from our collaborators at the University of Maryland (unpublished observations, Wu, H.Q. and Schwarcz, R.).

Effect of increased endogenous levels of KYNA on rule acquisition

This experiment examined whether increased KYNA levels results in a general cognitive impairment by impairing initial rule acquisition. Figure 5 represents performance of saline treated animals (n = 6) and kynurenine (50 mg/kg) treated animals (n = 6) on Day 1 of the set-shifting task. On average (mean ± SEM), saline treated animals required 36.5 ± 9.2 trials and kynurenine treated animals required 43.2 ± 9.7 trials to reach criterion. Therefore, this indicates that elevations in KYNA pre-Set 1 did not have a significant effect on the number of trials to reach criterion ($F_{1,11}$ = 1.491, P = 0.250). Furthermore, while the % correct varied over BLOCK ($F_{9,90}$ = 8.466, P < 0.001), they did not vary by BLOCK X GROUP ($F_{9,90}$ = 0.464, P =0.831) or GROUP ($F_{1,10}$ = 0.514, P = 0.490). Therefore, both the saline and kynurenine treated animals learned at a similar rate. Trials to criterion on Day 2 of testing for pre-Set 1 treated animals were analyzed using the Kruskal-Wallis test for

nonparametric data because of the imposed 80-trial cap in this test. On average (mean ± SEM), saline treated animals required 57.4 ± 7.7 trials and kynurenine treated animals required 47.4 ± 9.2 trials to reach criterion. Therefore, elevations in endogenous KYNA levels pre-Set 1 did not have a significant effect on the number of trials to required to reach criterion on Day 2 of testing (X^2 = 0.521, P = 0.470). Therefore, increased KYNA levels does not impair rule acquisition as no significant difference in the number of trials required to reach criterion on either Day 1 or Day 2 testing were found. Additionally, there was no difference in % correct starts from perseverative arms ($F_{1,11}$ = 0.144, P = 0.712) or reinforcement arms ($F_{1,11}$ = 0.403, P = 0.540) for kynurenine and saline treated animals.

Effect of increased endogenous levels of KYNA on performance in a cognitive flexibility task

This experiment examined whether increased KYNA levels results in impaired flexible responding. Figure 6 represents performance of saline treated animals (n = 7), kynurenine (50 mg/kg) treated animals (n = 7) that reached criterion (n = 2), and kynurenine treated animals that did not reach criterion (n = 5) on Day 2 of the set-shifting task. On average (mean ± SEM) for Day 1 of testing, saline treated animals required 39.4 ± 18.4 trials and kynurenine treated animals required 38.3 ± 10.6 trials to reach criterion. Therefore, this indicates that elevations in KYNA pre-Set 1 did not have a significant effect on the number of trials to reach criterion ($F_{1,13}$ = 0.020, P = 0.889). Trials to criterion in Set 2 were analyzed using the Kruskal-Wallis test for nonparametric data because of the imposed 80-trial cap in this test. On average (mean ± SEM) for Day 2 of testing, saline treated animals required 50.4 ± 5.0 trials and kynurenine treated animals required 56.3 ± 7.2 trials to reach criterion. While the % correct choices of Set 2 varied over BLOCK ($F_{9,108}$ = 5.508, P < 0.001), they did not differ based on drug treatments (BLOCK X GROUP; $F_{9,108}$ = 1.385, P = 0.227) nor did the two treatment groups vary from one another over the

duration of the task (GROUP; $F_{1,12}$ = 2.758, P = 0.123). Interestingly, elevations in endogenous KYNA levels pre-Set 2 did have a significant effect on the number of trials required to reach criterion on Day 2 of testing (X^2 = 4.545, P = 0.033) which reflects impairment on the EDS of the task. Additionally, there was no difference in percent correct starts from perseverative arms ($F_{1,13}$= 2.315, P = 0.154) or reinforcement arms ($F_{1,13}$ = 0.072, P = 0.793) for kynurenine and saline treated animals. Therefore, impairments were not due to an inability to abandon the previously reinforced stimuli.

DISCUSSION

The results of these experiments contributed further knowledge regarding the ability of KYNA to regulate chemotransmission and behavior. This study revealed two major findings. The first experiment demonstrated that (1) KYNA, and hence astrocytic activity, negatively modulates basal cortical ACh transmission (Figure 3). More importantly, the second experiment determined a functionally significant role for KYNA in cognitive processing as (2) elevated KYNA levels specifically impaired the ability of animals to perform an EDS that was not due to a general cognitive impairment as increasing levels of this metabolite did not impair rule acquisition (Figures 5,6). A discussion of the issues raised by each finding is included below.

KYNA to regulate basal cortical ACh transmission

This study demonstrates the ability of an astrocyte-derived metabolite, KYNA, to negatively modulate basal cortical cholinergic transmission. While this is the first study to reveal that astrocytic activity modulates cortical cholinergic transmission, it does not elucidate the precise molecular and cellular events that mediate its regulatory capacity. Additionally, it is extremely unlikely that this is a direct effect as there is no evidence for the

presence of _7nAChR autoreceptors on cholinergic terminals or interneurons in the PFC (Von Engelhardt et al., 2007). Therefore, the role of KYNA in the regulation of ACh transmission is most likely mediated through mechanisms that indirectly influence extracellular ACh release. Interestingly, the localization of _7nAChR is well documented on glutamatergic nerve terminals (Rassoulpour et al., 2005). Furthermore, _7nAChR activation is normally associated with direct facilitation of glutamatergic transmission in the brain (Gray et al., 1996; Fu et al., 2000). As such, the ability of KYNA to attenuate glutamatergic function *in vivo* (Pellicciaro et al., 1994; Harris et al., 1998; Cozzi et al., 1999; Schwarcz et al., 1999) can be attributed to its inhibitory action on _7nAChRs on glutamatergic terminals. Therefore, KYNA may bi-directionally regulate Glu release through its inhibitory actions on _7nAChRs located on glutamatergic terminals.

However, as previously mentioned, there is also a role for KYNA in the regulation of DA chemotransmission (Rassoulpour et al., 2005; Wu et al., 2007). As such, any proposed mechanism for regulatory roles of KYNA must explain how both neurotransmitter systems are simultaneously affected. In fact, it has been proposed that the _7nAChRs regulate glutamate release (Kaiser & Wonnacott, 2000), which in turn controls extracellular DA via a glutamate receptor subtype, α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA), situated on dopaminergic nerve terminals (Cheramy et al., 1986; Imperato et al., 1990; Leviel et al., 1990; Moghaddam & Bolinao, 1994; Borland & Micheal, 2004).

This sequential activity is consistent with demonstration that galantamine, which at low concentrations potentiates _7nAChRs allosterically (Santos et al., 2002; Sanochocki et al., 2003), prevents the KYNA-induced reduction of extracellular dopamine. Additionally, the ability of KYNA to influence extracellular DA levels likely involves its inhibitory action on _7nAChR rather than NMDA receptors. Evidence for this is derived from studies where the effect of KYNA on DA was not influenced by co-perfusion with D-serine, also an agonist of the glycine$_B$ receptor (Schell, 2004). Additionally, the KYNA-derivative, 7-Cl-KYNA, which is

a selective glycine$_B$ receptor antagonist, is incapable of influencing extracellular DA levels (Rassoulpour et al., 2005). Also, AMPA receptors are located on cholinergic neurons in several brain regions (Bernard et al., 1997; Bloomfield et al., 2007). Therefore, this would facilitate the ability of KYNA to indirectly influence ACh transmission.

Elevated KYNA levels impaired extra-dimensional shifting without impairing rule acquisition

We proposed that since schizophrenics present with elevated KYNA levels and impaired cognitive flexibility, increases in this metabolite may mediate this behavioral impairment. Interestingly, this was not due to a general cognitive impairment as increasing KYNA levels does not impair rule acquisition. These data present an interesting parallel to the human literature that indicates schizophrenics are impaired on ED shifting in the WCST (Robbins, 2007; Pantelis et al., 1999). Importantly, this maze-based set-shifting task was developed to reveal many of the same cognitive operations highlighted by the WCST (Birrell & Brown, 2000). Interestingly, neuroleptic treatment reduces KYNA levels suggesting a potential mechanism by which chronic antipsychotic treatment may alleviate some aspects of cognitive dysfunctions in patients (Ceresoli-Borrini et al., 2006).

Moreover, previous studies suggest additional roles for KYNA in other symptoms of schizophrenia. For instance, schizophrenics exhibit pronounced deficits in psychophysiological measures of sensory gating (Patterson et al., 2000). Moreover, both glutamatergic and cholinergic mechanisms are implicated in the neurobiological basis of auditory gating as administration of a noncompetitive NMDA receptor antagonist (MK-801; Miller et el., 1992) and _7nAChR antagonists (Freedman et al., 1997) disrupts auditory gating. Shepard et al. demonstrated that the association of elevated brain levels of KYNA in rats with auditory gating impairments was not attributable to the glutamate receptor antagonist properties of the metabolite (2003). In addition, prepulse inhibition (PPI) of the

acoustic startle reflex is an operational measure of sensorimotor gating that is reduced in schizophrenia (Braff et al., 1978). Erhardt et al. (2004) discovered that brain KYNA serves as an endogenous modulator of PPI, which is consistent with a hypothesis that aberrations in KYNA synthesis may contribute to various pathophysiologies of schizophrenia (2004).

While these studies suggest a role for KYNA mediated psychopathologies, a recent study has also demonstrated the ability for KYNA to impair cognitive functions like spatial working memory (Chess et al., 2007) that was not attributed to altered locomotor activity or motivation to consume food. Given this evidence, we proposed KYNA may also impair behavioral flexibility, an executive function disrupted in schizophrenia.

Although we discovered a role for KYNA in the regulation of cognitive flexibility, this may not be strictly mediated through a cholinergic mechanism as literature is mixed on the role of ACh in an attentional set-shifting task. One study that maintains ACh transmission is necessary to shift attention examined the effects of a muscarinic antagonist, scopolamine. Interestingly, systemic administration of scopolamine impairs EDS in the task (Chen et al., 2004). However, the central location of this cholinergic mechanism has yet to be specified.

On the other hand, a few recent studies suggest the role of cholinergic mechanisms in set shifting is not paramount to the EDS in this task. For instance, selective lesions of basal forebrain cholinergic neurons with 192-IgG-saporin did not result in impairments on shifting of an attentional set (Tait & Brown, 2008). Therefore, perhaps performance in this task is dependent upon a complex interaction amongst multiple neurotransmitter systems like DA, Glu, and ACh (Block et al., 2007). For example, systemic administration of the MK-801 impaired the EDS required of the task (Stefani & Moghaddam, 2005). Interestingly, this impairment is caused by an increased number of perseverative errors, in contrast to our findings. Additionally, Stefani & Moghaddam also examined the ability of MK-801 administration during a critical period of brain development to impair flexible responding (2005b). This period in rats is equivalent to the prenatal second trimester of primate

development and most neurodevelopmental models of schizophrenia focus on this postnatal period (Lipska et al., 2002). They discovered that a transient disruption of NMDA receptor function using MK-801 during this critical period impaired cognitive flexibility due to increased perseverative behavior. Furthermore, acute injections of phencyclidine (PCP) induced EDS deficits in an attentional set-shifting task, and moreover, atypical antipsychotics significantly improved the PCP-induced cognitive deficit (McLean et al., 2008). Also, in addition to NMDA receptor mediated impairments in flexible responding, AMPA receptor antagonists also impaired EDS. However, this effect can be distinguished from MK-801 induced deficits in the EDS of the task because it produces a more general cognitive deficit (Stefani et al., 2003). Therefore, these studies support a KYNA based targeting of the Glu system in impairments seen in the present study.

In addition, dopaminergic neurotransmission in the cortex also plays a role in set-shifting behavior as shown in laboratory animals (Floresco et al., 2006; Stefani & Moghaddam, 2006) and human volunteers (Mehta et al., 2004). Moreover, cortical (Laplante et al., 2004; Del Arco et al., 2007) and NAC (Zmarowski et al., 2005; Brooks et al., 2007) dopamine receptor activity can also regulate cortical ACh levels.

Collectively, this literature provides a framework in which to view and understand our observations that KYNA impaired performance in a set-shifting task. Unfortunately, the literature conflicts regarding the relative contribution of cholinergic mechanisms mediating cognitive flexibility; however, their likely contribution cannot be completely dismissed as ACh transmission may be faciliatory for DA and Glu-mediated functioning that appears integral for this task. Additionally, the critical neural structures for cholinergic involvement in set-shifting may comprise distributed systems and not be restricted to those within the neocortex (Chen et al., 2004).

FUTURE DIRECTIVES

Currently, studies are underway to determine if bilateral, intra-cortical perfusions of L-KYN or KYNA will also impair flexible responding in a perceptual set-shifting task. We predict that both of these manipulations will do so as a systemic injection of L-KYN also impaired the ability to perform extra-dimensional shifts in the task. However, local manipulations will provide greater validity and reinforce the conclusions generated from the present experiments. In addition, it would be interesting to examine whether set-shifting deficits were attenuated by co-administration of typical or atypical neuroleptics as chronic treatment with these are known to reduce levels of KYNA. Moreover, attempts should be made to dissociate the contribution of cholinergic versus glutamatergic transmission in an attentional set-shifting task.

CONCLUSIONS

The present study is both innovative and significant because this is the first study to reveal that astrocytic activity modulates cortical cholinergic transmission. This research also demonstrated a role for KYNA in the regulation of prefrontally-mediated cognitive flexibility. Collectively, these studies may help uncover a role for KYNA-ACh dysfunction in schizophrenia, which could facilitate the development of more efficacious pharmaceuticals utilized for the treatment of cognitive deficits seen in neuropsychiatric disorders.

FIGURES AND FIGURE LEGENDS

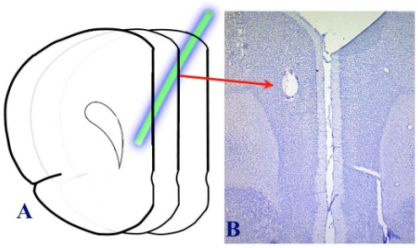

Figure 1. Representative histology for mPFC probe placements. (A) A schematic depiction of an mPFC placement. Guides were implanted so that when probes were inserted, the membrane tip (3.0 mm) was located at AP +4.2, LM +0.6, DV -0.6 from dura pointing 20° rostral. (B) A representative photomicrograph from a subject with the mPFC placement that is illustrated in (A). Due to the angle at which the guide cannula and probe were positioned, the arrow points to the site of termination of the dialysis probe. All coordinates were based upon Paxinos and Watson (1998).

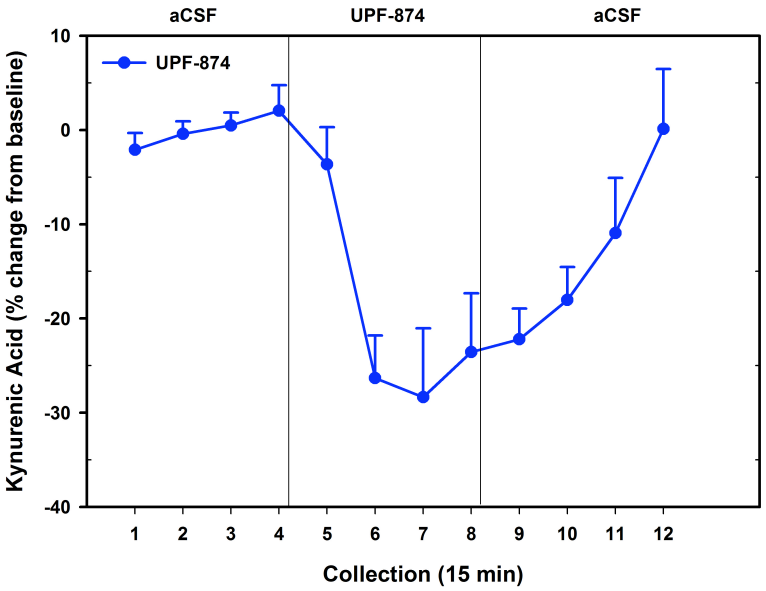

Figure 2. Mean (± SEM) kynurenic acid (KYNA) efflux in the mPFC of animals (n = 5) receiving vehicle (artificial cerebral spinal fluid, aCSF) or a moderate concentration of a kynurenine aminotransferase II inhibitor UPF-874 alone (5 mM) into the mPFC over the course of one microdialysis session. Following a 3-h washout period, four baseline samples were collected (collections 1-4). This was followed by the administration of aCSF + drug (vehicle, UPF-874). After the 60 min perfusion of aCSF + drug (collections 5-8), aCSF alone was perfused for 60 min (collections 9-12) until the end of the microdialysis period. UPF-874 alone significantly decreased KYNA efflux below vehicle. Data from this experiment was gathered from our collaborators at the University of Maryland (unpublished observations, Wu, H.Q. and Schwarcz, R.).

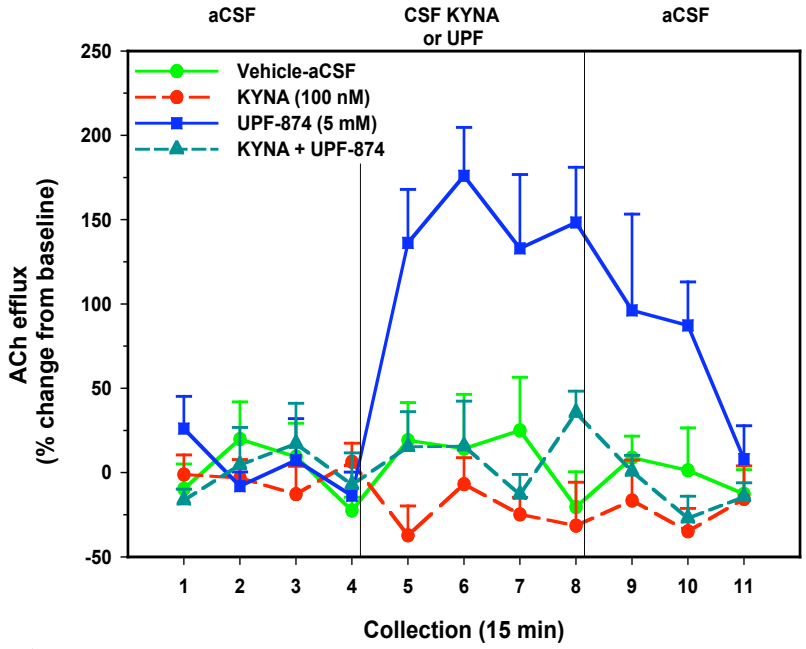

Figure 3. Mean (± SEM) acetylcholine (ACh) efflux in the mPFC of animals (n = 8) receiving, in counterbalanced order, vehicle (artificial cerebrospinal fluid, aCSF), a kynurenine aminotransferase II inhibitor UPF 874 alone (5 mM), a physiologically relevant concentration of kynurenic acid (KYNA) alone (100 nM), and co-administration of UPF 874 + KYNA into the mPFC during four separate microdialysis sessions. Following a 3-h washout period, four baseline samples were collected (collections 1-4). This was followed by the administration of aCSF + drug (vehicle, KYNA, UPF 874, or KYNA + UPF 874) for 60 min (collections 5-8). After the 60 min perfusion of aCSF + drug, aCSF alone was perfused for 45 min until the end of the microdialysis period (collections 9-11). KYNA alone reduced ACh efflux below vehicle. UPF 874 alone significantly increased ACh efflux above vehicle. Co-administration of KNYA and UPF 874 resulted in an ACh efflux similar to that produced by vehicle alone. Therefore, the increase in ACh efflux produced by UPF 874 was blocked by concurrent perfusion of KYNA.

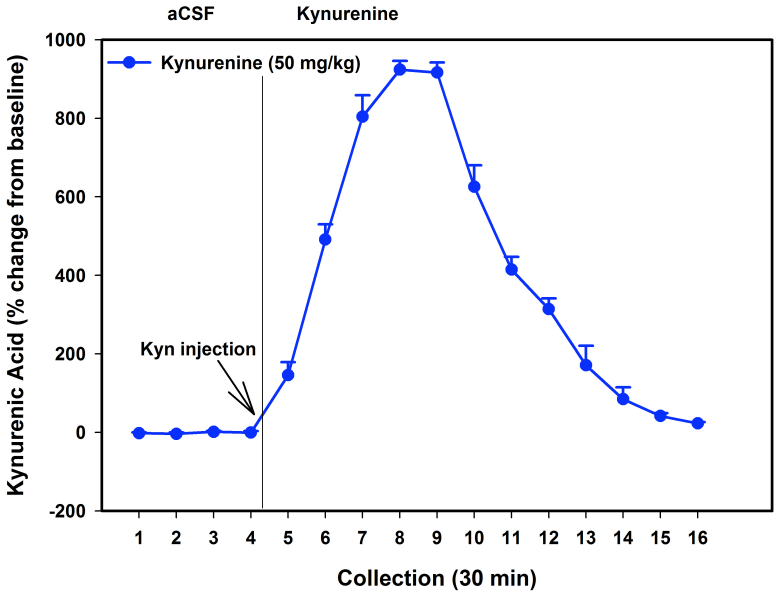

Figure 4. Mean (± SEM) kynurenic acid (KYNA) efflux in the mPFC of animals (n = 5) receiving a systemic injection of kynurenine (50 mg/kg). Vehicle (artificial cerebral spinal fluid, aCSF) was constantly perfused through the microdialysis probe in the mPFC. Following a 3-h washout period, four baseline samples were collected (collections 1-4). This was followed by a systemic injection of kynurenine after which multiple collections (5-16) were taken to allow KYNA levels to return to baseline. Following the injection, a max increase in KYNA was observed approximately 45 min later. Data from this experiment was gathered from our collaborators at the University of Maryland (unpublished observations, Wu, H.Q. and Schwarcz, R.).

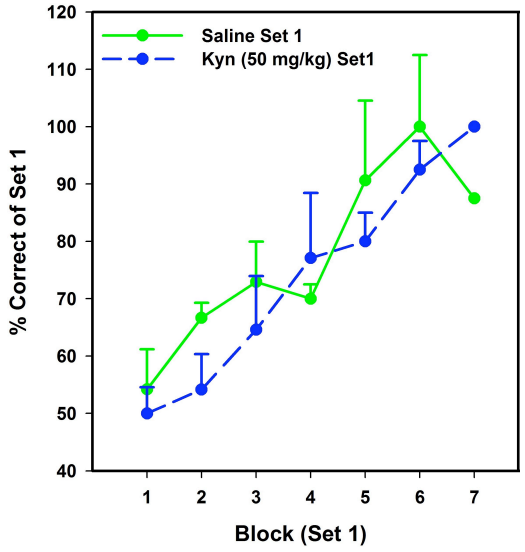

Figure 5. Mean (± SEM) % correct responses by Block for the acquisition of the Set 1 testing process as represented by Blocks. Each Block represents 8 trials (80 total trials). Therefore, early-task performance is represented by % correct responses during Blocks 1-3, mid-task performance is reflected by % correct responses during Blocks 4-7, and end-task performance is indicated by % correct responses during Blocks 8-10. Both the saline treated rats (control; n = 6) and the kynurenine (50 mg/kg) treated rats (n = 6) learn the new rule as indicated by the acquisition curve or gradual improvement in responding over time. Both groups learned the new rule, at a similar rate, and by Block 7 successfully reached criterion, 8 consecutive correct trials.

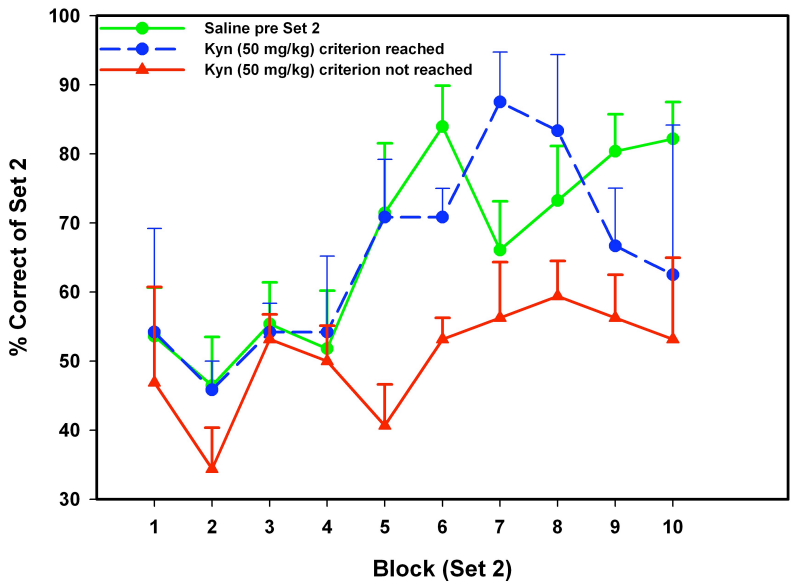

Figure 6. Mean (± SEM) % correct responses by Block for the acquisition of the Set 2 testing process as represented by Blocks. Each Block represents 8 trials (80 total trials). Therefore, early-task performance is represented by % correct responses during Blocks 1-3, mid-task performance is reflected by % correct responses during Blocks 4-7, and end-task performance is indicated by % correct responses during Blocks 8-10. The kynurenine (50 mg/kg) treated animals (n = 7) were subdivided into those that reached criterion (n = 2) and those that did not reach criterion (n = 5). All groups began the Set 2 testing process achieving a correct response to the new stimulus of approximately 50% because of responding to the previous rule. The kynurenine treated animals that did reach criterion performed at a similar rate to the saline treated animals and both groups learned the new rule as indicated by the acquisition curve. However, the kynurenine treated animals that did not reach criterion were unsuccessful in shifting behavioral response strategies as there is no indication of an acquisition curve over time. They consistently achieved a correct response about %50 of the time.

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